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(71) Applicant: **Ajinomoto Co., Ltd.**
Tokyo (JP)

(72) Inventors:
- **Izui, Hiroshi c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Ono, Eiji c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Matsui, Kazuhiko c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Moriya, Mika c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Ito, Hisao c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Hara, Yoshihiko c/o Ajinomoto Co.,Inc.**
  Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)

(74) Representative: **Strehl Schübel-Hopf & Partner**
Maximilianstrasse 54
80538 München (DE)

(54) L-glutamic acid-producing bacterium and method for producing l-glutamic acid

(57) L-Glutamic acid is produced by culturing in a liquid culture medium a microorganism belonging to the genus *Enterobacter* or *Serratia* and having an ability to produce L-glutamic acid, which increases in an activity of enzyme catalyzing a reaction for L-glutamic acid biosynthesis, or which decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid, and collecting produced L-glutamic acid from the culture medium.

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Description

BACKGROUND OF THE INVENTION

[0001] The present invention relates to a novel L-glutamic acid-producing bacterium and a method for producing L-glutamic acid by fermentation using the same. L-Glutamic acid is an important amino acid as food, drugs and the like.

[0002] L-Glutamic acid has conventionally been produced by fermentation methods utilizing the so-called coryneform L-glutamic acid-producing bacteria which principally belong to the genera *Brevibacterium, Corynebacterium,* and *Microbacterium* or variants thereof ("Amino Acid Fermentation", Gakkai Shuppan Center, pp.195-215, 1986). As methods for producing L-glutamic acid by fermentation utilizing other bacterial strains, there have been known the methods utilizing microorganisms of the genera *Bacillus, Streptomyces, Penicillium* and the like (Unite States Patent No. 3,220,929), the methods utilizing microorganisms of the genera *Pseudomonas, Arthrobacter, Serratia, Candida* and the like (Unite States Patent No. 3,563,857), the methods utilizing microorganisms of the genera *Bacillus, Pseudomonas, Serratia* and the like or *Aerobacter aerogenes* (currently referred to as *Enterobacter aerogenes)* (Japanese Patent Publication (KOKOKU) No. 32-9393(1957)), the method utilizing variant strains of *Escherichia coli* (Japanese Patent Application Laid-Open (KOKAI) No. 5-244970(1993)) and the like.

[0003] Though the productivity of L-glutamic acid has considerably been improved by breeding of such microorganisms as mentioned above or improvements of production methods, it is still desired to develop a more inexpensive and more efficient method for producing L-glutamic acid in order to meet the expected markedly increasing future demand of the amino acid.

SUMMARY OF THE INVENTION

[0004] The object of the present invention is to find a novel L-glutamic acid-producing bacterium having a high ability to produce L-glutamic acid, thereby developing a more inexpensive and more efficient method for producing L-glutamic acid.

[0005] To achieve the aforementioned object, the present inventors intensively searched for and studied microorganisms having the ability to produce L-glutamic acid that are different from the previously reported microorganisms. As a result, they found that certain strains derived from microorganisms belonging to the genus *Enterobacter* or *Serratia* had a high ability to produce L-glutamic acid, and have completed the present invention.

[0006] Thus, the present invention provide:

(1) a microorganism belonging to the genus *Enterobacter* or *Serratia* and having an ability to produce L-glutamic acid and at least one of the following properties:

(a) the microorganism increases in an activity of an enzyme catalyzing a reaction for L-glutamic acid biosynthesis; and
(b) the microorganism decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid;

(2) a microorganism of the above (1) wherein the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis is at least one selected from the group consisting of citrate synthase (abbreviated as "CS" hereinafter), phosphoenolpyruvate carboxylase (abbreviated as "PEPC" hereinafter), and glutamate dehydrogenase (abbreviated as "GDH" hereinafter);

(3) a microorganism of the above (2) wherein the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis includes all of CS, PEPC and GDH;

(4) a microorganism of any one of the above (1) to (3) wherein the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid is α-ketoglutarate dehydrogenase (abbreviated as "aKGDH" hereinafter);

(5) a microorganism of any one of the above (1) to (4) which is *Enterobacter agglomerans* or *Serratia liquefacience;* and

(6) a method for producing L-glutamic acid which comprises culturing the microorganism as defined in any one of the above (1) to (5) in a liquid culture medium to produce and accumulate L-glutamic acid in the culture medium, and collecting the L-glutamic acid from the culture medium.

[0007] Because the microorganism of the present invention have a high ability to produce L-glutamic acid, it is considered to be possible to impart a further higher production ability to the microorganism by using the breeding techniques previously known for th coryneform L-glutamic acid-producing bacteria and the like, and it is expected to contribute

to development of a more inexpensive and more efficient method for producing L-glutamic acid by appropriately selecting culture conditions and the like.

BRIEF EXPLANATION OF THE DRAWINGS

[0008] Figure 1 shows construction of a plasmid pMWCPG having a *gltA* gene, a *ppc* gene and a *gdhA* gene.

[0009] Figure 2 shows construction of a plasmid pSTVG having the *gdhA* gene.

[0010] Figure 3 shows construction of a plasmid RSF-Tet having a replication origin of a wide-host-range plasmid RSF1010 and a tetracycline resistance gene.

[0011] Figure 4 shows construction of a plasmid RSFCPG having the replication origin of the wide-host-range plasmid RSF1010, the tetracycline resistance gene, the *gltA* gene, the *ppc* gene and the *gdhA* gene.

[0012] Figure 5 shows construction of a plasmid pMWCB having the *gltA* gene.

[0013] Figure 6 shows a restriction map of a DNA fragment of pTWVEK101 derived from *Enterobacter agglomerans*.

[0014] Figure 7 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucA* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*. The upper sections: *Enterobacter agglomerans*, the lower sections: *Escherichia coli* (the same shall apply hereinafter).

[0015] Figure 8 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucB* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.

[0016] Figure 9 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sdhB* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.

[0017] Figure 10 shows comparison of an amino acid sequence deduced from a nucleotide sequence of a *sucC* gene derived from *Enterobacter agglomerans* with one derived from *Escherichia coli*.

DETAILED DESCRIPTION OF THE INVENTION

[0018] The present invention will be explained in detail hereinafter.

[0019] The microorganism of the present invention is a microorganism belonging to the genus *Enterobacter* or *Serratia*, and having at least one of the following properties:

(a) the microorganism increases in an activity of an enzyme catalyzing a reaction for L-glutamic acid biosynthesis; and

(b) the microorganism decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid.

[0020] Such a microorganism can be obtained by using a microorganism belonging to the genus *Enterobacter* or the genus *Serratia* as a parent strain, and imparting the properties of the above (a) and/or (b) to the microorganism. Examples of the microorganism belonging to the genus *Enterobacter* or the genus *Serratia* that can be used as the parent strain are listed below:

Enterobacter agglomerans
Enterobacter aerogenes
Enterobacter amnigenus
Enterobacter asburiae
Enterobacter cloacae
Enterobacter dissolvens
Enterobacter gergoviae
Enterobacter hormaechei
Enterobacter intermedius
Enterobacter nimipressuralis
Enterobacter sakazakii
Enterobacter taylorae
Serratia liquefacience
Serratia entomophila
Serratia ficaria
Serratia fonticola
Serratia grimesii
Serratia proteamaculans
Serratia odorifera

Serratia plymuthica
Serratia rubidaea

[0021] More preferably, those bacterial strains listed below can be mentioned:

Enterobacter agglomerans ATCC 12287
Enterobacter agglomerans AJ13355
Serratia liquefacience ATCC 14460

[0022] The *Enterobacter agglomerans* AJ13355 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, and received an accession number of FERM P-16644, and then transferred to an international deposition under the Budapest Treaty on January 11, 1999, and received an accession number of FERM BP-6614. The *Enterobacter agglomerans* ATCC 12287, and the *Serratia liquefacience* ATCC 14460 are available from ATCC.

[0023] The *Enterobacter agglomerans* AJ13355 is a strain isolated from soil in Iwata-shi, Shizuoka, Japan.

[0024] Physiological properties of AJ13355 are as follows:

(1) Gram stain: Negative
(2) Behavior for oxygen: Facultative anaerobe
(3) Catalase: Negative
(4) Oxidase: Positive
(5) Nitrate reduction: Negative
(6) Voges-Proskauer reaction: Positive
(7) Methyl Red test: Negative
(8) Urease: Negative
(9) Indole production: Positive
(10) Motility: Present
(11) Hydrogen sulfide production in TSI culture medium: Slightly active
(12) β-Galactosidase: Positive
(13) Sugar assimilability:

Arabinose: Positive
Sucrose: Positive
Lactose: Positive
Xylose: Positive
Sorbitol: Positive
Inositol: Positive
Trehalose: Positive
Maltose: Positive
Melibiose: Positive
Adonitol: Negative
Raffinose: Positive
Salicin: Negative
Melibiose: Positive

(14) Glycerose assimilability: Positive
(15) Organic acid assimilability:

Citric acid: Positive
Tartaric acid: Negative
Gluconic acid: Positive
Acetic acid: Positive
Malonic acid: Negative

(16) Arginine dehydratase: Negative
(17) Ornithine decarboxylase: Negative
(18) Lysine decarboxylase: Negative
(19) Phenylalanine deaminase: Negative

(20) Pigment formation: Yellow
(21) Gelatin liquefaction: Positive
(22) Growth pH: Not good growth at pH 4, good growth at pH 4.5 to 7
(23) Growth temperature: Good growth at 25°C, good growth at 30°C, good growth at 37°C, growth possible at 42°C, no growth at 45°C

[0025] From these bacteriological properties, AJ13355 is determined to be *Enterobacter agglomerans*.

[0026] In the working examples described hereinafter, *Enterobacter agglomerans* ATCC12287, *Enterobacter agglomerans* AJ13355, and *Serratia liquefacience* ATCC14460 were used as starting parent strains for obtaining strains which increase in the activity of the enzyme catalyzing the reactions for the L-glutamic acid biosynthesis, or strains which decrease in or are deficient in the activity of the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid. However, the sugar metabolism by any of bacteria belonging to the genera *Enterobacter* and *Serratia is* achieved via the Embden-Meyerhof pathway, and pyruvate produced in the pathway is oxidized in the tricarboxylic acid cycle under aerobic conditions. L-Glutamic acid is biosynthesized from α-ketoglutaric acid which is an intermediate of the tricarboxylic acid cycle by GDH or glutamine synthetase/glutamate synthase. Thus, these microorganisms share the same biosynthetic pathway for L-glutamic acid, and microorganism belonging to the genera *Enterobacter* and *Serratia* are encompassed within a single concept according to the present invention. Therefore, microorganisms belonging to the genera *Enterobacter* and *Serratia* other than species and strains specifically mentioned above also fall within the scope of the present invention.

[0027] The microorganism of the present invention is a microorganism belonging to the genus *Enterobacter* or the genus *Serratia* and having an ability to produce L-glutamic acid. The expression "having an ability to produce L-glutamic acid" as herein used means to have an ability to accumulate L-glutamic acid in a culture medium during cultivation. According to the present invention, the ability to produce L-glutamic acid is imparted by giving either one or both of the following characteristics:

(a) the microorganism increases in the activity of the enzyme catalyzing the reaction for the L-glutamic acid biosynthesis; and
(b) the microorganism decreases in or is deficient in the activity of the enzyme catalyzing the reaction branching from the pathway for L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid.

[0028] As examples of the enzyme catalyzing the reaction for L-glutamic acid biosynthesis of microorganisms of the genus *Enterobacter* or *Serratia,* there can be mentioned GDH, glutamine synthetase, glutamate synthase, isocitrate dehydrogenase, aconitate hydratase, CS, PEPC, pyruvate dehydrogenase, pyruvate kinase, enolase, phosphoglyceromutase, phosphoglycerate kinase, glyceraldehyde-3-phosphate dehydrogenase, triosephosphate isomerase, fructose bisphosphate aldolase, phosphofructokinase, glucose phosphate isomerase and the like. Among these enzymes, one or two or three kinds of CS, PEPC and GDH are preferred. As for the microorganism of the present invention, it is further preferred that activities of all of the three kinds of enzymes, CS, PEPC and GDH, are increased. Whether a microorganism increases in an activity of a target enzyme, and degree of the increase of the activity can be determined by measuring the enzyme activity of a bacterial cell extract or a purified fraction, and comparing it with that of a wild type strain or a parent strain.

[0029] The microorganism of the present invention, which belongs to the genus *Enterobacter* or *Serratia,* and increases in the activity of the enzyme catalyzing the reaction for L-glutamic acid biosynthesis, can be obtained as, for example, a variant where mutation has been made in a gene encoding the enzyme or a genetic recombinant strain by using any of the microorganisms mentioned above as a starting parent strain.

[0030] To enhance the activity of CS, PEPC or GDH, for example, a gene encoding CS, PEPC or GDH can be cloned in a suitable plasmid, and the aforementioned starting parent strain as a host can be transformed with the resulting plasmid. This can increase the copy number of each of the genes encoding CS, PEPC and GDH (hereinafter abbreviated as *"gltA* gene", *"ppc* gene", and *"gdhA* gene", respectively), and as a result the activities of CS, PEPC and GDH can be increased.

[0031] One or two or three kinds selected from the cloned *gltA* gene, *ppc* gene and *gdhA* gene in any combination are introduced into the starting parent strain mentioned above. When two or three kinds of the genes are introduced, either the two or three kinds of the genes are cloned in one kind of plasmid, and introduced into the host, or they are separately cloned in two or three kinds of plasmids that can exist in the same host, and introduced into the host.

[0032] The plasmid is not particularly limited so long as it can autonomously replicate in a microorganism belonging to the genus *Enterobacter* or *Serratia.* Examples of the plasmid include, for example, pUC19, pUC18, pBR322, pHSG299, pHSG298, pHSG399, pHSG398, RSF1010, pMW119, pMW118, pMW219, pMW218 and the like. Other than these plasmids, phage DNA vectors can also be utilized.

[0033] Transformation can be achieved by, for example, the method of D.M. Morrison (Methods in Enzymology 68,

326 (1979)), the method by increasing permeability of recipient cells for DNA with calcium chloride (Mandel, M. and Higa, A., J. Mol. Biol., 53, 159 (1970)), or the like.

[0034] The activities of CS, PEPC and GDH can also be increased by using multiple copies of the *gltA* gene, the *ppc* gene and/or the *gdh* gene present on the chromosome DNA of the starting parent strain as a host. In order to introduce multiple copies of the *gltA* gene, the *ppc* gene and/or the gdhA gene into a chromosome DNA of a microorganism belonging to the genus *Enterobacter* or *Serratia*, sequences present on chromosome DNA in a multiple copy number such as repetitive DNA, and inverted repeats present at an end of transposition factors can be utilized. Alternatively, multiple copies of the genes can also be introduced into a chromosome DNA by utilizing transposition of transposons carrying the *gltA* gene, the *ppc* gene, or the *gdhA* gene. These techniques can increase the copy number of the *gltA* gene, the *ppc* gene, and the *gdhA* gene in transformant cells, and as a result increase the activities of CS, PEPC and GDH.

[0035] Any organisms can be used as a source of the *gltA* gene, the *ppc* gene and the *gdhA* gene used for increasing copy numbers, so long as the organisms have the CS, PEPC and GDH activities. Among such organisms, bacteria, i. e., prokaryotes, such as those bacteria belonging to the genera *Enterobacter, Klebsiella, Erwinia, Pantoea, Serratia, Escherichia, Corynebacterium, Brevibacterium,* and *Bacillus* are preferred. As a specific example, *Escherichia coli* can be mentioned. The *gltA* gene, the *ppc* gene and the *gdhA* gene can be obtained from a chromosome DNA of such microorganisms as mentioned above.

[0036] The *gltA* gene, the *ppc* gene and the *gdhA* gene can each be obtained from a chromosome DNA of any of the aforementioned microorganisms by isolating a DNA fragment complementing auxotrophy of a variant strain lacking the CS, PEPC or GDH activity. Alternatively, because the nucleotide sequences of these genes of bacteria of the genus *Escherichia* or *Corynebacterium* have already been elucidated (Biochemistry, Vol. 22, pp.5243-5249, 1983; J. Biochem. Vol. 95, pp.909-916, 1984; Gene, Vol. 27, pp.193-199, 1984; Microbiology, Vol. 140, pp.1817-1828, 1994; Mol. Gen. Genet. Vol. 218, pp.330-339, 1989; and Molecular Microbiology, Vol. 6, pp.317-326, 1992), the genes can be obtained by PCR using primers synthesized based on each of the elucidated nucleotide sequences, and the chromosome DNA as a template.

[0037] The activity of CS, PEPC or GDH can also be increased by, other than by the gene amplification mentioned above, enhancing expression of the *gltA* gene, the *ppc* gene or the *gdhA* gene. For example, the expression is enhanced by replacing the promoter of the *gltA* gene, the *ppc* gene, or the *gdhA* gene with another stronger promoter. Examples of such a strong promoter include, for example, a *lac* promoter, a *trp* promoter, a *trc* promoter, a *tac* promoter, a P_R promoter and a P_L promoter of lambda phage and the like. The *gltA* gene, the *ppc* gene, or the *gdhA* gene of which promoter has been substituted is cloned into a plasmid and introduced into a host microorganism, or introduced into a chromosome DNA of host microorganism using a repetitive DNA, inverted repeat, transposon or the like.

[0038] The activities of CS, PEPC or GDH can also be increased by replacing the promoter of the *gltA* gene, the *ppc* gene, or the *gdhA* gene on a chromosome with another stronger promoter (see WO87/03006, and Japanese Patent Application Laid-Open (KOKAI) No. 61-268183(1986)), or inserting a strong promoter at the upstream of each coding sequence of the genes (see Gene, 29, pp. 231-241, 1984). Specifically, these are achieved by homologous recombination between the *gltA* gene, the *ppc* gene, or the *gdhA* gene of which promoter is replaced with a stronger promoter or DNA containing a part of them, and a corresponding gene on the chromosome.

[0039] Specific examples of the microorganism belonging to the genus *Enterobacter* or *Serratia* of which CS, PEPC or GDH activity is increased include, for example, *Enterobacter agglomerans* ATCC12287/RSFCPG, *Enterobacter agglomerans* AJ13355/RSFCPG, and *Serratia liquefacience* ATCC14460/RSFCPG.

[0040] Examples of the enzyme catalyzing the reaction branching from the pathway of L-glutamic acid biosynthesis and producing the compound other than L-glutamic acid include, for example, αKGDH, isocitrate lyase, phosphate acetyltransferase, acetate kinase, acetohydroxy acid synthase, acetolactate synthase, formate acetyltransferase, lactate dehydrogenase, glutamate decarboxylase, 1-pyrroline dehydrogenase and the like. Among these enzymes, αKGDH is preferred.

[0041] In order to obtain such decrease or deficiency of enzyme activity as mentioned above in a microorganism belonging to the genus *Enterobacter* or *Serratia*, a mutation causing the decrease or deficiency of the enzyme activity can be introduced into a gene encoding the enzyme by a conventional mutagenesis technique or genetic engineering technique.

[0042] Examples of the mutagenesis technique include, for example, the method utilizing irradiation of X-ray or ultraviolet light, the method utilizing treatment with a mutagenic agent such as N-methyl-N'-nitro-N-nitrosoguanidine and the like. The site of gene to which a mutation is introduced may be a coding region encoding an enzyme protein, or an expression regulatory region such as a promoter.

[0043] Examples of the genetic engineering technique include, for example, genetic recombination, genetic transduction, cell fusion and the like. For example, a drug resistance gene is inserted into a target gene to produc a functionally inactivated gene (defective gene). Then, this defective gene is introduced into a cell of a microorganism belonging to the genus *Enterobacter* or Serratia, and the target gene on a chromosome is replaced with the defectiv

gene by homologous recombination (gene disruption).

[0044] Whether a microorganism decreases in an activity of a target enzyme or is deficient in the activity, and degree of the decrease of the activity can be determined by measuring the enzyme activity of a bacterial cell extract or a purified fraction of a candidate strain, and comparing it with that of a wild type strain or a parent strain. The αKGDH enzymatic activity can be measured by, for example, the method of Reed et al. (L.J. Reed and B.B. Mukherjee, Methods in Enzymology 1969, 13, p.55-61).

[0045] Depending on the target enzyme, a target variant can be selected based on a phenotype of the variant. For example, a variant which is deficient in the aKGDH activity or decreases in the activity cannot grow on a minimal medium containing glucose, or a minimal medium containing acetic acid or L-glutamic acid as an exclusive carbon source, or shows markedly reduced growth rate therein under aerobic conditions. However, even under the same condition, it can exhibit normal growth by addition of succinic acid or lysine, methionine and diaminopimelate to the minimal medium containing glucose. Based on these phenomena, a variant that is deficient in the αKGDH activity or decreases in the activity can be selected.

[0046] A method for producing a *Brevibacterium lactofermentum* strain lacking the αKGDH gene based on homogenous recombination is detailed in WO95/34672, and a similar method can be used for microorganisms belonging to the genera *Enterobacter* and *Serratia*.

[0047] In addition, procedures of genetic cloning, cleavage and ligation of DNA, transformation and the like are detailed in Molecular Cloning, 2nd edition, Cold Spring Harbor Press (1989) and the like.

[0048] An example of the variant strain that is deficient in the αKGDH activity or decreases in the activity obtained as described above is *Enterobacter agglomerans* AJ13356. The *Enterobacter agglomerans* AJ13356 was deposited at the National Institute of Bioscience and Human-Technology, Agency of Industrial Science and Technology, Ministry of International Trade and Industry on February 19, 1998, received an accession number of FERM P-16645, and then transferred to an international deposition under the Budapest Treaty on January 11, 1999, and received an accession number of FERM BP-6615.

[0049] The microorganism belonging to the genus *Enterobacter* or *Serratia,* and having at least one of the properties (a) and (b) and an ability to produce L-glutamic acid can be cultured in a liquid medium to produce and accumulate L-glutamic acid in the medium.

[0050] The culture medium may be an ordinary nutrient medium containing a carbon source, a nitrogen source, and inorganic salts, as well as organic trace nutrients such as amino acids, vitamins and the like, as required. It can be a synthetic medium or a natural medium. Any carbon sources and nitrogen sources can be used for the culture medium so long as they can be utilized by the microorganism to be cultured.

[0051] The carbon source may be a saccharide such as glucose, glycerol, fructose, sucrose, maltose, mannose, galactose, starch hydrolysates, molasses and the like. Further, an organic acid such as acetic acid and citric acid may also be used alone or in combination with other carbon sources.

[0052] The nitrogen source may be ammonia, ammonium salts such as ammonium sulfate, ammonium carbonate, ammonium chloride, ammonium phosphate, and ammonium acetate, nitrates and the like.

[0053] As organic trace nutrients, amino acids, vitamins, fatty acids, nucleic acids, materials containing them such as peptone, casamino acid, yeast extract, and soybean protein decomposition products and the like are used, and when an auxotrophic variant which requires an amino acid or the like for its growth is used, it is necessary to complement the nutrient required.

[0054] As the inorganic salt, phosphates, magnesium salts, calcium salts, iron salts, manganese salts and the like are used.

[0055] As for the culture conditions, cultivation may be performed under aerobic conditions at a temperature of 20 to 42°C and a pH of 4 to 8. The cultivation can be continued for 10 hours to 4 days to accumulate a considerable amount of L-glutamic acid in the liquid culture medium.

[0056] After the completion of the cultivation, L-glutamic acid accumulated in the culture medium may be collected by a known method. For example, it can be isolated by a method comprising concentrating the medium after removing the cells to crystallize the product, ion exchange chromatography or the like.

Examples

[0057] The present invention will be explained more specifically with reference to the following examples.

(1) Construction of plasmid having *gltA* gene, *ppc* gene and *gdhA* gene

[0058] Procedure for construction of a plasmid having a *gltA* gene, a *ppc* gene and a *gdhA* gene will be explained by referring to Figure 1 to Figure 5.

[0059] A plasmid pBRGDH having a *gdhA* gene derived from *Escherichia coli* (Japanese Patent Application Laid-

Open (KOKAI) No. 7-203980(1995)) was digested with HindIII and *SphI,* and the both ends were blunt-ended by a treatment with T4 DNA polymerase. Then, a DNA fragment containing the *gdhA* gene was purified and collected. On the other hand, a plasmid pMWCP having a *gltA* gene and a *ppc* gene derived from *Escherichia coli* (WO97/08294) was digested with *XbaI,* and the both ends were blunt-ended by a treatment with T4 DNA polymerase. This was mixed with the DNA fragment having the *gdhA* gene purified above, and ligated with T4 ligase, giving a plasmid pMWCPG, which corresponds to the pMWCP further carrying the *gdhA* gene (Figure 1).

[0060] A DNA fragment having the *gdhA* gene obtained by digesting the pBRGDH with HindIII and *SalI* was purified and collected, and introduced into the *HindIII-SalI* site of a plasmid pSTV29 (purchased from Takara Shuzo) to obtain a plasmid pSTVG (Figure 2).

[0061] At the same time, a product obtained by digesting a plasmid pVIC40 having a replication origin of a wide-host-range plasmid RSF1010 (Japanese Patent Application Laid-Open (KOKAI) No. 8-047397(1996)) with *NotI,* followed by T4 DNA polymerase treatment and *PstI* digestion, and a product obtained by digesting pBR322 with EcoT141, followed by T4 DNA polymerase treatment and *PstI* digestion, were mixed and ligated with T4 ligase to obtain a plasmid RSF-Tet having the replication origin of RSF1010 and a tetracycline resistance gene (Figure 3).

[0062] Then, the pMWCPG was digested with EcoRI and *PstI,* and a DNA fragment having the *gltA* gene, the *ppc* gene and the *gdhA* gene was purified and collected. Similarly, the RSF-Tet was digested with *EcoRI* and *PstI,* and a DNA fragment having the replication origin of RSF1010 was purified and collected. Those DNA fragments were mixed and ligated with T4 ligase to obtain a plasmid RSFCPG composed of RSF-Tet carrying the *gltA* gene, the *ppc* gene and the *gdhA* gene (Figure 4). Expression of the *gltA* gene, the *ppc* gene and the *gdhA* gene by the resulting plasmid RSFCPG, and expression of the *gdhA* gene by the pSTVG were confirmed based on complementation of auxotrophy of *Escherichia coli* strains lacking the *gltA* gene, the *ppc* gene or the *gdhA* gene, and measurement of each enzyme activity.

[0063] A plasmid having a *gltA* gene derived from *Brevibacterium lactofermentum* was constructed as follows. PCR was performed by using primers having the nucleotide sequences represented in SEQ ID NOS: 6 and 7 selected based on the nucleotide sequence of the *gltA* gene of *Corynebacterium glutamicum* (Microbiology, 140, 1817-1828, 1994), and a chromosome DNA of *Brevibacterium lactofermentum* ATCC 13869 as a template to obtain a *gltA* gene fragment of about 3 kb. This fragment was inserted into a plasmid pHSG399 (purchased from Takara Shuzo) digested with *SmaI* to obtain a plasmid pHSGCB (Figure 5). Then, the pHSGCB was digested with HindIII, and an excised *gltA* gene fragment of about 3 kb was inserted into a plasmid pMW218 (purchased from Nippon Gene) digested with HindIII to obtain a plasmid pMWCB (Figure 5). Expression of the *gltA* gene by the resulting plasmid pMWCB was confirmed by determination of enzyme activity in the *Enterobacter agglomerans* AJ13355 .

(2) Introduction of RSFCPG, pMWCB and pSTVG into *Enterobacter agglomerans* or *Serratia liquefacience,* and evaluation of L-glutamic acid productivity

[0064] The *Enterobacter agglomerans* ATCC 12287, the *Enterobacter agglomerans* AJ13355 and the *Serratia liquefacience* ATCC 14460 were transformed with the RSFCPG, pMWCB and pSTVG by electroporation (Miller J.H., "A Short Course in Bacterial Genetics; Handbook" Cold Spring Harbor Laboratory Press, USA, 1992) to obtain transformants exhibiting tetracycline resistance.

[0065] Each of the resulting transformants and the parent strains was inoculated into 50 ml-volume large size test tube containing 5 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, and 30 g/L calcium carbonate, and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. However, as for the AJ13355/pMWCB strain and the AJ13355/pSTVG strain, the cultivation was stopped when about 10 g/L of glucose was consumed, i.e., cultivated for 15 hours like the parent strain AJ13355, because their glucose consumption rates were low. To the culture medium of the transformants, 25 mg/L of tetracycline was added. After the cultivation was completed, L-glutamic acid accumulated in the culture medium was measured. The results are shown in Table 1.

Table 1:

Accumulated amount of L-glutamic acid	
Bacterial strain	Accumulated amount of L-glutamic acid
ATCC12287	0.0 g/L
ATCC12287/RSFCPG	6.1

Table 1: (continued)

Accumulated amount of L-glutamic acid	
Bacterial strain	Accumulated amount of L-glutamic acid
AJ13355	0.0
AJ13355/RSFCPG	3.3
AJ13355/pMWCB	0.8
AJ13355/pSTVG	0.8
ATCC14460	0.0
ATCC14460/RSFCPG	2.8
Culture medium alone	0.2

[0066] While the *Enterobacter agglomerans* ATCC12287, the *Enterobacter agglomerans* AJ13355 and the *Serratia liquefacience* ATCC14460 did not accumulate L-glutamic acid, the strains whose CS, PEPC and GDH activities were amplified by introducing RSFCPG accumulated 6.1 g/L, 3.3 g/L, and 2.8 g/L of L-glutamic acid, respectively. The AJ13355 strain of which CS activity alone was amplified accumulated 0.8 g/L of L-glutamic acid, and the strain of which GDH activity alone was amplified also accumulated 0.8 g/L of L-glutamic acid.

(3) Cloning of αKGDH gene (referred to as *"sucAB"* hereinafter) of *Enterobacter agglomerans* AJ13355

[0067] The *sucAB* gene of the *Enterobacter agglomerans* AJ13355 was cloned by selecting a DNA fragment complementing acetate non-assimilation of an *Escherichia coli* strain lacking the αKGDH-E1 subunit gene (referred to as *"sucA"* hereinafter) from the chromosome DNA of the *Enterobacter agglomerans* AJ13355.

[0068] The chromosome DNA of the *Enterobacter agglomerans* AJ13355 strain was isolated by the same method as conventionally used for extracting chromosome DNA from *Escherichia coli* (Seibutsu Kogaku Jikken-sho (Textbook of Bioengineering Experiments), Ed. by the Society of Fermentation and Bioengineering, Japan, p.97-98, Baifukan, 1992). The pTWV228 used as the vector (ampicillin resistant) was a marketed product from Takara Shuzo.

[0069] Products obtained by digesting the chromosome DNA of the AJ13355 strain with *EcoT221* and products obtained by digesting the pTWV228 with *PstI* were ligated by T4 ligase, and the *Escherichia coli* JRG465 lacking *sucA* (Herbert J. et al., Mol. Gen. Genetics, 1969, 105, p.182) was transformed with them. Strains grown on the acetic acid minimal medium were selected from the transformants obtained as described above, and a plasmid extracted from them was designated as pTWVEK101. The *Escherichia coli* JRG465 carrying the pTWVEK101 recovered the characteristics of acetate non-assimilability as well as auxotrophy for succinic acid or L-lysine and L-methionine. This suggests that the pTWVEK101 contains the *sucA* gene of *Enterobacter agglomerans*.

[0070] A restriction map of *Enterobacter agglomerans*-derived DNA fragment of pTWVEK101 is shown in Figure 6. The result of nucleotide sequencing of the hatched portion in Figure 6 is shown in SEQ ID NO: 1. In this sequence, two full length ORFs and two nucleotides sequences considered as partial sequences of ORFs were found. Amino acid sequences that can be encoded by these ORFs and the partial sequences thereof are shown in SEQ ID NOS: 2 to 5 in order from the 5' ends. As a result of homology analysis of these sequences, it was found that the portion of which nucleotide sequence had been determined contained a 3' partial sequence of succinate dehydrogenase iron-sulfur protein gene *(sdhB)*, full length *sucA* and αKGDH-E2 subunit gene *(sucB* gene), and 5' partial sequence of succinyl-CoA synthetase β subunit gene *(sucC* gene). Comparison of the amino acid sequences deduced from these nucleotide sequences with those of *Escherichia coli.* (Eur. J. Biochem., 141, 351-359 (1984), Eur. J. Biochem., 141, 361-374 (1984), and Biochemistry, 24, 6245-6252 (1985)) is shown in Figures 7 to 9. As shown by these results, the amino acid sequences exhibited markedly high homology. It was also found that a cluster of *sdhB-sucA-sucB-sucC* is formed on the *Enterobacter agglomerans* chromosome like *Escherichia coli* (Eur. J. Biochem., 141, 351-359 (1984), Eur. J. Biochem., 141, 361-374 (1984), and Biochemistry, 24, 6245-6252 (1985)).

(4) Acquisition of strain deficient in αKGDH derived from *Enterobacter agglomerans* AJ13355

[0071] Using the *sucAB* gene of *Enterobacter agglomerans* obtained as described above, a strain lacking αKGDH of *Enterobacter agglomerans* was obtained by homologous recombination.

[0072] First, pTWVEK101 was digested with *BglII* to remove the C-terminus region corresponding to about half of the *sucA* gene and the full length of the *sucB* gene. To this site, a chloramphenicol resistance gene fragment cut out from the pHSG399 (Takara Shuzo) with AccI was then inserted. The region of *sdhB-ΔsucAB::Cm^r-sucC* obtained above was cut out with *AflII* and SacI. The resulting DNA fragment was used to transform the *Enterobacter agglomerans*

AJ13355 strain by electroporation to obtain a chloramphenicol resistant strain, and thus a *Enterobacter agglomerans* AJ13356 strain lacking the *sucAB* gene where the *sucAB* gene on the chromosome was replaced by *sucAB*::Cm^r was obtained.

[0073] To confirm that the AJ13356 strain obtained as described above was deficient in the αKGDH activity, its enzymatic activity was determined by the method of Reed (L.J. Reed and B.B. Mukherjee, Methods in Enzymology 1969, 13, p.55-61). As a result, the αKGDH activity could not be detected in the AJ13356 strain as shown in Table 2, and thus it was confirmed that the strain lacked the *sucAB* as desired.

Table 2:

αKGDH activity	
Bacterial strain	αKGDH activity (ΔABS/min/mg protein)
AJ13355	0.481
AJ13356	<0.0001

(5) Evaluation of L-glutamic acid productivity of *Enterobacter agglomerans* strain deficient in aKGDH

[0074] Each of the AJ13355 and AJ13356 strains was inoculated into a 500 ml-volume flask containing 20 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, 30 g/L calcium carbonate, 200 mg/L L-lysine monohydrochloride, 200 mg/L L-methionine and 200 mg/L DL-α,ε-diaminopimelic acid (DAP), and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. After the cultivation was completed, L-glutamic acid and α-ketoglutaric acid (abbreviated as "αKG" hereinafter) accumulated in the culture medium were measured. The results are shown in Table 3.

Table 3:

Accumulated amounts of L-glutamic acid and αKG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of αKG
AJ13355	0.0 g/L	0.0 g/L
AJ13356	1.5	3.2

[0075] The AJ13356 strain deficient in the aKGDH activity accumulated 1.5 g/L of L-glutamic acid, and simultaneously accumulated 3.2 g/L of αKG.

(6) Introduction of RSFCPG into *Enterobacter agglomerans* strain lacking αKGDH and evaluation of L-glutamic acid productivity

[0076] The AJ13356 strain was transformed with the RSFCPG, and the resulting strain introduced with the RSFCPG, AJ13356/RSFCPG, was inoculated into a 500 ml-volume flask containing 20 ml of a culture medium comprising 40 g/L glucose, 20 g/L ammonium sulfate, 0.5 g/L magnesium sulfate heptahydrate, 2 g/L potassium dihydrogenphosphate, 0.5 g/L sodium chloride, 0.25 g/L calcium chloride heptahydrate, 0.02 g/L ferrous sulfate heptahydrate, 0.02 g/L manganese sulfate tetrahydrate, 0.72 mg/L zinc sulfate dihydrate, 0.64 mg/L copper sulfate pentahydrate, 0.72 mg/L cobalt chloride hexahydrate, 0.4 mg/L boric acid, 1.2 mg/L sodium molybdate dihydrate, 2 g/L yeast extract, 25 mg/L tetracycline, 30 g/L calcium carbonate, 200 mg/L L-lysine monohydrochloride, 200 mg/L L-methionine and 200 mg/L DL-α,ε-DAP, and cultured at 37°C with shaking until the glucose contained in the culture medium was consumed. After the cultivation was completed, L-glutamic acid and αKG accumulated in the culture medium were measured. The results are shown in Table 4.

Table 4:

Accumulated amounts of L-glutamic acid and αKG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of αKG
AJ13356	1.4 g/L	2.9 g/L

Table 4: (continued)

Accumulated amounts of L-glutamic acid and αKG		
Bacterial strain	Accumulated amount of L-glutamic acid	Accumulated amount of αKG
AJ13356/RSFCPG	5.1	0.0

[0077] In the strain of which CS, PEPC and GDH activities were amplified by the introduction of RSFCPG, the accumulated amount of αKG was reduced, and the accumulated amount of L-glutamic acid was further improved.

Annex to the description

[0078]

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930                 935                       1                 5
att ctc gtt ccc gac ctg cct gaa tcg gtt gca gat gcc aca gta gca   3207
Ile Leu Val Pro Asp Leu Pro Glu Ser Val Ala Asp Ala Thr Val Ala
                 10                  15                  20
acc tgg cac aag aaa cca ggc gat gca gtc agc cgc gat gaa gtc atc   3255
Thr Trp His Lys Lys Pro Gly Asp Ala Val Ser Arg Asp Glu Val Ile
             25                  30                  35
gtc gaa att gaa act gac aaa gtc gtg ctg gaa gtg ccg gca tct gcc   3303
Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu Val Pro Ala Ser Ala
         40                  45                  50
gat ggc gtg ctg gaa gcc gtg ctg gaa gac gaa ggg gca acc gtt acg   3351
Asp Gly Val Leu Glu Ala Val Leu Glu Asp Glu Gly Ala Thr Val Thr
     55                  60                  65
tcc cgc cag atc ctg ggt cgc ctg aaa gaa ggc aac agt gcg ggt aaa   3399
Ser Arg Gln Ile Leu Gly Arg Leu Lys Glu Gly Asn Ser Ala Gly Lys
```

```
 70                  75                  80                  85
gaa agc agt gcc aaa gcg gaa agc aat gac acc acg cca gcc cag cgt   3447
Glu Ser Ser Ala Lys Ala Glu Ser Asn Asp Thr Thr Pro Ala Gln Arg
                 90                  95                 100
cag aca gcg tcg ctt gaa gaa gag agc agc gat gcg ctc agc ccg gcg   3495
Gln Thr Ala Ser Leu Glu Glu Glu Ser Ser Asp Ala Leu Ser Pro Ala
            105                 110                 115
atc cgt cgc ctg att gcg gag cat aat ctt gac gct gcg cag atc aaa   3543
Ile Arg Arg Leu Ile Ala Glu His Asn Leu Asp Ala Ala Gln Ile Lys
        120                 125                 130
ggc acc ggc gta ggc gga cgt tta acg cgt gaa gac gtt gaa aaa cat   3591
Gly Thr Gly Val Gly Gly Arg Leu Thr Arg Glu Asp Val Glu Lys His
    135                 140                 145
ctg gcg aac aaa ccg cag gct gag aaa gcc gcc gcg cca gcg gcg ggt   3639
Leu Ala Asn Lys Pro Gln Ala Glu Lys Ala Ala Ala Pro Ala Ala Gly
150                 155                 160                 165
gca gca acg gct cag cag cct gtt gcc aac cgc agc gaa aaa cgt gtt   3687
Ala Ala Thr Ala Gln Gln Pro Val Ala Asn Arg Ser Glu Lys Arg Val
                170                 175                 180
ccg atg acg cgt tta cgt aag cgc gtc gcg gag cgt ctg ctg gaa gcc   3735
Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu Arg Leu Leu Glu Ala
            185                 190                 195
aag aac agc acc gcc atg ttg acg acc ttc aac gaa atc aac atg aag   3783
Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn Glu Ile Asn Met Lys
        200                 205                 210
ccg att atg gat ctg cgt aag cag tac ggc gat gcg ttc gag aag cgt   3831
Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Asp Ala Phe Glu Lys Arg
    215                 220                 225
cac ggt gtg cgt ctg ggc ttt atg tct ttc tac atc aag gcc gtg gtc   3879
His Gly Val Arg Leu Gly Phe Met Ser Phe Tyr Ile Lys Ala Val Val
230                 235                 240                 245
gaa gcg ctg aag cgt tat cca gaa gtc aac gcc tct atc gat ggc gaa   3927
Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala Ser Ile Asp Gly Glu
                250                 255                 260
gac gtg gtg tac cac aac tat ttc gat gtg agt att gcc gtc tct acg   3975
Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser Ile Ala Val Ser Thr
            265                 270                 275
cca cgc gga ctg gtg acg cct gtc ctg cgt gac gtt gat gcg ctg agc   4023
Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp Val Asp Ala Leu Ser
        280                 285                 290
atg gct gac atc gag aag aaa att aaa gaa ctg gca gtg aaa ggc cgt   4071
Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu Ala Val Lys Gly Arg
    295                 300                 305
gac ggc aag ctg acg gtt gac gat ctg acg ggc ggt aac ttt acc atc   4119
Asp Gly Lys Leu Thr Val Asp Asp Leu Thr Gly Gly Asn Phe Thr Ile
310                 315                 320                 325
acc aac ggt ggt gtg ttc ggt tcg ctg atg tct acg cca atc atc aac   4167
```

```
Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser Thr Pro Ile Ile Asn
                330                 335                 340
ccg cca cag agc gcg att ctg ggc atg cac gcc att aaa gat cgt cct   4215
Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala Ile Lys Asp Arg Pro
            345                 350                 355
atg gcg gtc aat ggt cag gtt gtg atc ctg cca atg atg tac ctg gct   4263
Met Ala Val Asn Gly Gln Val Val Ile Leu Pro Met Met Tyr Leu Ala
        360                 365                 370
ctc tcc tac gat cac cgt tta atc gat ggt cgt gaa tct gtc ggc tat   4311
Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg Glu Ser Val Gly Tyr
    375                 380                 385
ctg gtc gcg gtg aaa gag atg ctg gaa gat ccg gcg cgt ctg ctg ctg   4359
Leu Val Ala Val Lys Glu Met Leu Glu Asp Pro Ala Arg Leu Leu Leu
390                 395                 400                 405
gat gtc tgattcatca ctgggcacgc gttgcgtgcc caatctcaat actcttttca   4415
Asp Val

gatctgaatg gatagaacat c atg aac tta cac gaa tac cag gct aaa cag   4466
                        Met Asn Leu His Glu Tyr Gln Ala Lys Gln
                          1               5                  10
ctg ttt gca cgg tat ggc atg cca gca ccg acc ggc tac gcc tgt act   4514
Leu Phe Ala Arg Tyr Gly Met Pro Ala Pro Thr Gly Tyr Ala Cys Thr
                    15                  20                  25
aca cca cgt gaa gca gaa gaa gcg gca tcg aaa atc ggt gca           4556
Thr Pro Arg Glu Ala Glu Glu Ala Ala Ser Lys Ile Gly Ala
              30                  35                  40

<210> 2
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<213> Enterobacter agglomerans
<400> 2
Ala Phe Ser Val Phe Arg Cys His Ser Ile Met Asn Cys Val Ser Val
  1               5                  10                  15
Cys Pro Lys Gly Leu Asn Pro Thr Arg Ala Ile Gly His Ile Lys Ser
             20                  25                  30
Met Leu Leu Gln Arg Ser Ala
         35

<210> 3
<211> 935
<212> PRT
<213> Enterobacter agglomerans
<400> 3
Met Gln Asn Ser Ala Met Lys Pro Trp Leu Asp Ser Ser Trp Leu Ala
  1               5                  10                  15
Gly Ala Asn Gln Ser Tyr Ile Glu Gln Leu Tyr Glu Asp Phe Leu Thr
```

```
                20                  25                  30
Asp Pro Asp Ser Val Asp Ala Val Trp Arg Ser Met Phe Gln Gln Leu
        35                  40                  45
Pro Gly Thr Gly Val Lys Pro Glu Gln Phe His Ser Ala Thr Arg Glu
    50                  55                  60
Tyr Phe Arg Arg Leu Ala Lys Asp Ala Ser Arg Tyr Thr Ser Ser Val
65                  70                  75                  80
Thr Asp Pro Ala Thr Asn Ser Lys Gln Val Lys Val Leu Gln Leu Ile
                85                  90                  95
Asn Ala Phe Arg Phe Arg Gly His Gln Glu Ala Asn Leu Asp Pro Leu
            100                 105                 110
Gly Leu Trp Lys Gln Asp Arg Val Ala Asp Leu Asp Pro Ala Phe His
        115                 120                 125
Asp Leu Thr Asp Ala Asp Phe Gln Glu Ser Phe Asn Val Gly Ser Phe
    130                 135                 140
Ala Ile Gly Lys Glu Thr Met Lys Leu Ala Asp Leu Phe Asp Ala Leu
145                 150                 155                 160
Lys Gln Thr Tyr Cys Gly Ser Ile Gly Ala Glu Tyr Met His Ile Asn
                165                 170                 175
Asn Thr Glu Glu Lys Arg Trp Ile Gln Gln Arg Ile Glu Ser Gly Ala
            180                 185                 190
Ser Gln Thr Ser Phe Ser Gly Glu Glu Lys Lys Gly Phe Leu Lys Glu
        195                 200                 205
Leu Thr Ala Ala Glu Gly Leu Glu Lys Tyr Leu Gly Ala Lys Phe Pro
    210                 215                 220
Gly Ala Lys Arg Phe Ser Leu Glu Gly Gly Asp Ala Leu Val Pro Met
225                 230                 235                 240
Leu Arg Glu Met Ile Arg His Ala Gly Lys Ser Gly Thr Arg Glu Val
                245                 250                 255
Val Leu Gly Met Ala His Arg Gly Arg Leu Asn Val Leu Ile Asn Val
            260                 265                 270
Leu Gly Lys Lys Pro Gln Asp Leu Phe Asp Glu Phe Ser Gly Lys His
        275                 280                 285
Lys Glu His Leu Gly Thr Gly Asp Val Lys Tyr His Met Gly Phe Ser
    290                 295                 300
Ser Asp Ile Glu Thr Glu Gly Gly Leu Val His Leu Ala Leu Ala Phe
305                 310                 315                 320
Asn Pro Ser His Leu Glu Ile Val Ser Pro Val Val Met Gly Ser Val
                325                 330                 335
Arg Ala Arg Leu Asp Arg Leu Ala Glu Pro Val Ser Asn Lys Val Leu
            340                 345                 350
Pro Ile Thr Ile His Gly Asp Ala Ala Val Ile Gly Gln Gly Val Val
        355                 360                 365
Gln Glu Thr Leu Asn Met Ser Gln Ala Arg Gly Tyr Glu Val Gly Gly
    370                 375                 380
Thr Val Arg Ile Val Ile Asn Asn Gln Val Gly Phe Thr Thr Ser Asn
385                 390                 395                 400
```

```
Pro Lys Asp Ala Arg Ser Thr Pro Tyr Cys Thr Asp Ile Gly Lys Met
                405                 410                 415
Val Leu Ala Pro Ile Phe His Val Asn Ala Asp Asp Pro Glu Ala Val
            420                 425                 430
Ala Phe Val Thr Arg Leu Ala Leu Asp Tyr Arg Asn Thr Phe Lys Arg
        435                 440                 445
Asp Val Phe Ile Asp Leu Val Cys Tyr Arg Arg His Gly His Asn Glu
    450                 455                 460
Ala Asp Glu Pro Ser Ala Thr Gln Pro Leu Met Tyr Gln Lys Ile Lys
465                 470                 475                 480
Lys His Pro Thr Pro Arg Lys Ile Tyr Ala Asp Arg Leu Glu Gly Glu
                485                 490                 495
Gly Val Ala Ser Gln Glu Asp Ala Thr Glu Met Val Asn Leu Tyr Arg
            500                 505                 510
Asp Ala Leu Asp Ala Gly Glu Cys Val Val Pro Glu Trp Arg Pro Met
        515                 520                 525
Ser Leu His Ser Phe Thr Trp Ser Pro Tyr Leu Asn His Glu Trp Asp
    530                 535                 540
Glu Pro Tyr Pro Ala Gln Val Asp Met Lys Arg Leu Lys Glu Leu Ala
545                 550                 555                 560
Leu Arg Ile Ser Gln Val Pro Glu Gln Ile Glu Val Gln Ser Arg Val
                565                 570                 575
Ala Lys Ile Tyr Asn Asp Arg Lys Leu Met Ala Glu Gly Glu Lys Ala
            580                 585                 590
Phe Asp Trp Gly Gly Ala Glu Asn Leu Ala Tyr Ala Thr Leu Val Asp
        595                 600                 605
Glu Gly Ile Pro Val Arg Leu Ser Gly Glu Asp Ser Gly Arg Gly Thr
    610                 615                 620
Phe Phe His Arg His Ala Val Val His Asn Gln Ala Asn Gly Ser Thr
625                 630                 635                 640
Tyr Thr Pro Leu His His Ile His Asn Ser Gln Gly Glu Phe Lys Val
                645                 650                 655
Trp Asp Ser Val Leu Ser Glu Glu Ala Val Leu Ala Phe Glu Tyr Gly
            660                 665                 670
Tyr Ala Thr Ala Glu Pro Arg Val Leu Thr Ile Trp Glu Ala Gln Phe
        675                 680                 685
Gly Asp Phe Ala Asn Gly Ala Gln Val Val Ile Asp Gln Phe Ile Ser
    690                 695                 700
Ser Gly Glu Gln Lys Trp Gly Arg Met Cys Gly Leu Val Met Leu Leu
705                 710                 715                 720
Pro His Gly Tyr Glu Gly Gln Gly Pro Glu His Ser Ser Ala Arg Leu
                725                 730                 735
Glu Arg Tyr Leu Gln Leu Cys Ala Glu Gln Asn Met Gln Val Cys Val
            740                 745                 750
Pro Ser Thr Pro Ala Gln Val Tyr His Met Leu Arg Arg Gln Ala Leu
        755                 760                 765
Arg Gly Met Arg Arg Pro Leu Val Val Met Ser Pro Lys Ser Leu Leu
```

```
    770                 775                 780
Arg His Pro Leu Ala Ile Ser Ser Leu Asp Glu Leu Ala Asn Gly Ser
785                 790                 795                 800
Phe Gln Pro Ala Ile Gly Glu Ile Asp Asp Leu Asp Pro Gln Gly Val
                805                 810                 815
Lys Arg Val Val Leu Cys Ser Gly Lys Val Tyr Tyr Asp Leu Leu Glu
            820                 825                 830
Gln Arg Arg Lys Asp Glu Lys Thr Asp Val Ala Ile Val Arg Ile Glu
        835                 840                 845
Gln Leu Tyr Pro Phe Pro His Gln Ala Val Gln Glu Ala Leu Lys Ala
    850                 855                 860
Tyr Ser His Val Gln Asp Phe Val Trp Cys Gln Glu Glu Pro Leu Asn
865                 870                 875                 880
Gln Gly Ala Trp Tyr Cys Ser Gln His His Phe Arg Asp Val Val Pro
                885                 890                 895
Phe Gly Ala Thr Leu Arg Tyr Ala Gly Arg Pro Ala Ser Ala Ser Pro
            900                 905                 910
Ala Val Gly Tyr Met Ser Val His Gln Gln Gln Gln Gln Asp Leu Val
        915                 920                 925
Asn Asp Ala Leu Asn Val Asn
    930                 935
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<210> 4
<211> 407
<212> PRT
<213> Enterobacter agglomerans
<400> 4

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  1               5                  10                  15
Asp Ala Thr Val Ala Thr Trp His Lys Lys Pro Gly Asp Ala Val Ser
            20                  25                  30
Arg Asp Glu Val Ile Val Glu Ile Glu Thr Asp Lys Val Val Leu Glu
        35                  40                  45
Val Pro Ala Ser Ala Asp Gly Val Leu Glu Ala Val Leu Glu Asp Glu
    50                  55                  60
Gly Ala Thr Val Thr Ser Arg Gln Ile Leu Gly Arg Leu Lys Glu Gly
65                  70                  75                  80
Asn Ser Ala Gly Lys Glu Ser Ser Ala Lys Ala Glu Ser Asn Asp Thr
                85                  90                  95
Thr Pro Ala Gln Arg Gln Thr Ala Ser Leu Glu Glu Glu Ser Ser Asp
            100                 105                 110
Ala Leu Ser Pro Ala Ile Arg Arg Leu Ile Ala Glu His Asn Leu Asp
        115                 120                 125
Ala Ala Gln Ile Lys Gly Thr Gly Val Gly Gly Arg Leu Thr Arg Glu
    130                 135                 140
Asp Val Glu Lys His Leu Ala Asn Lys Pro Gln Ala Glu Lys Ala Ala
145                 150                 155                 160
```

```
Ala Pro Ala Ala Gly Ala Ala Thr Ala Gln Gln Pro Val Ala Asn Arg
                165                 170                 175
Ser Glu Lys Arg Val Pro Met Thr Arg Leu Arg Lys Arg Val Ala Glu
            180                 185                 190
Arg Leu Leu Glu Ala Lys Asn Ser Thr Ala Met Leu Thr Thr Phe Asn
        195                 200                 205
Glu Ile Asn Met Lys Pro Ile Met Asp Leu Arg Lys Gln Tyr Gly Asp
    210                 215                 220
Ala Phe Glu Lys Arg His Gly Val Arg Leu Gly Phe Met Ser Phe Tyr
225                 230                 235                 240
Ile Lys Ala Val Val Glu Ala Leu Lys Arg Tyr Pro Glu Val Asn Ala
                245                 250                 255
Ser Ile Asp Gly Glu Asp Val Val Tyr His Asn Tyr Phe Asp Val Ser
            260                 265                 270
Ile Ala Val Ser Thr Pro Arg Gly Leu Val Thr Pro Val Leu Arg Asp
        275                 280                 285
Val Asp Ala Leu Ser Met Ala Asp Ile Glu Lys Lys Ile Lys Glu Leu
    290                 295                 300
Ala Val Lys Gly Arg Asp Gly Lys Leu Thr Val Asp Asp Leu Thr Gly
305                 310                 315                 320
Gly Asn Phe Thr Ile Thr Asn Gly Gly Val Phe Gly Ser Leu Met Ser
                325                 330                 335
Thr Pro Ile Ile Asn Pro Pro Gln Ser Ala Ile Leu Gly Met His Ala
            340                 345                 350
Ile Lys Asp Arg Pro Met Ala Val Asn Gly Gln Val Val Ile Leu Pro
        355                 360                 365
Met Met Tyr Leu Ala Leu Ser Tyr Asp His Arg Leu Ile Asp Gly Arg
    370                 375                 380
Glu Ser Val Gly Tyr Leu Val Ala Val Lys Glu Met Leu Glu Asp Pro
385                 390                 395                 400
Ala Arg Leu Leu Leu Asp Val
                405
```

<210> 5
<211> 40
<212> PRT
<213> Enterobacter agglomerans

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<400> 5
Met Asn Leu His Glu Tyr Gln Ala Lys Gln Leu Phe Ala Arg Tyr Gly
  1               5                  10                  15
Met Pro Ala Pro Thr Gly Tyr Ala Cys Thr Thr Pro Arg Glu Ala Glu
             20                  25                  30
Glu Ala Ala Ser Lys Ile Gly Ala
         35                  40
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<210> 6
<211> 30

<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: primer
<400> 6
gtcgacaata gccygaatct gttctggtcg 30

<210> 7
<211> 30
<212> DNA
<213> Artificial Sequence
<220>
<223> Description of Artificial Sequence: primer
<400> 7
aagcttatcg acgctcccct ccccaccgtt 30

Claims

1. A microorganism belonging to the genus Enterobacter or Serratia having the ability to produce L-glutamic acid and having at least one of the following properties:

   (a) the microorganism has increased activity of an enzyme catalyzing a reaction in the L-glutamic acid biosynthesis; and
   (b) the microorganism has decreased acitivity or is deficient in an activity of an enzyme catalyzing a reaction branching from the pathway of the L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid.

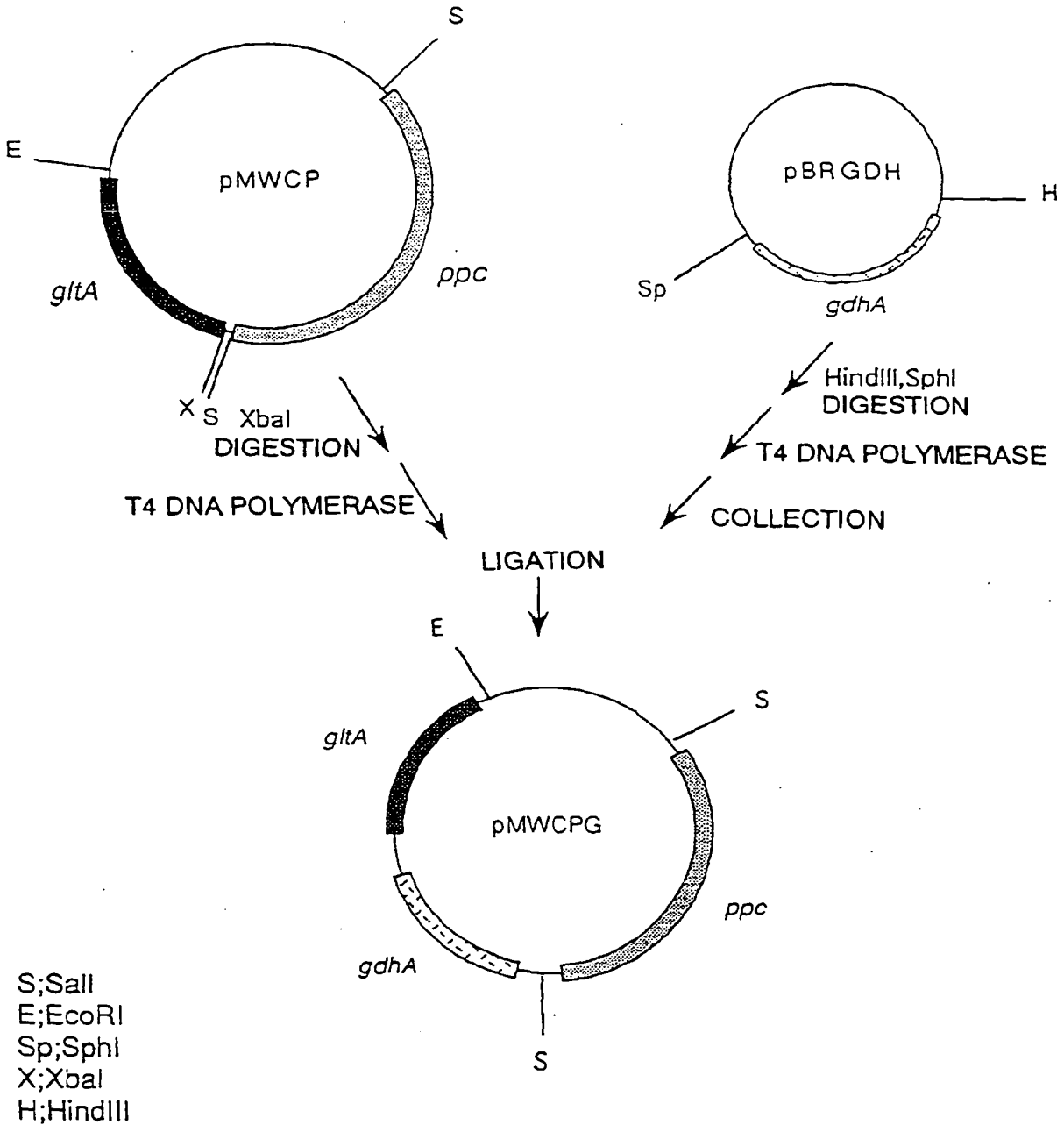
2. A microorganism according to claim 1 wherein the enzyme catalyzing the reaction in the L-glutamic acid biosynthesis is at least one selected from the group consisting of citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase.

3. A microorganism according to claim 2 wherein the enzyme catalyzing the reaction in the L-glutamic acid biosynthesis includes all of citrate synthase, phosphoenolpyruvate carboxylase, and glutamate dehydrogenase.

4. A microorganism according to any one of claims 1 to 3 wherein the enzyme catalyzing the reaction branching from the pathway of the L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid is α-ketoglutarate dehydrogenase.

5. A microorganism according to any one of claims 1 to 4 which is Enterobacter agglomerans or Serratia liquefaciens.

6. A method for producing L-glutamic acid which comprises culturing the microorganism as defined in any one of claims 1 to 5 in a liquid culture medium to produce and accumulate L-glutamic acid in the culture medium, and collecting the L-glutamic acid from the culture medium.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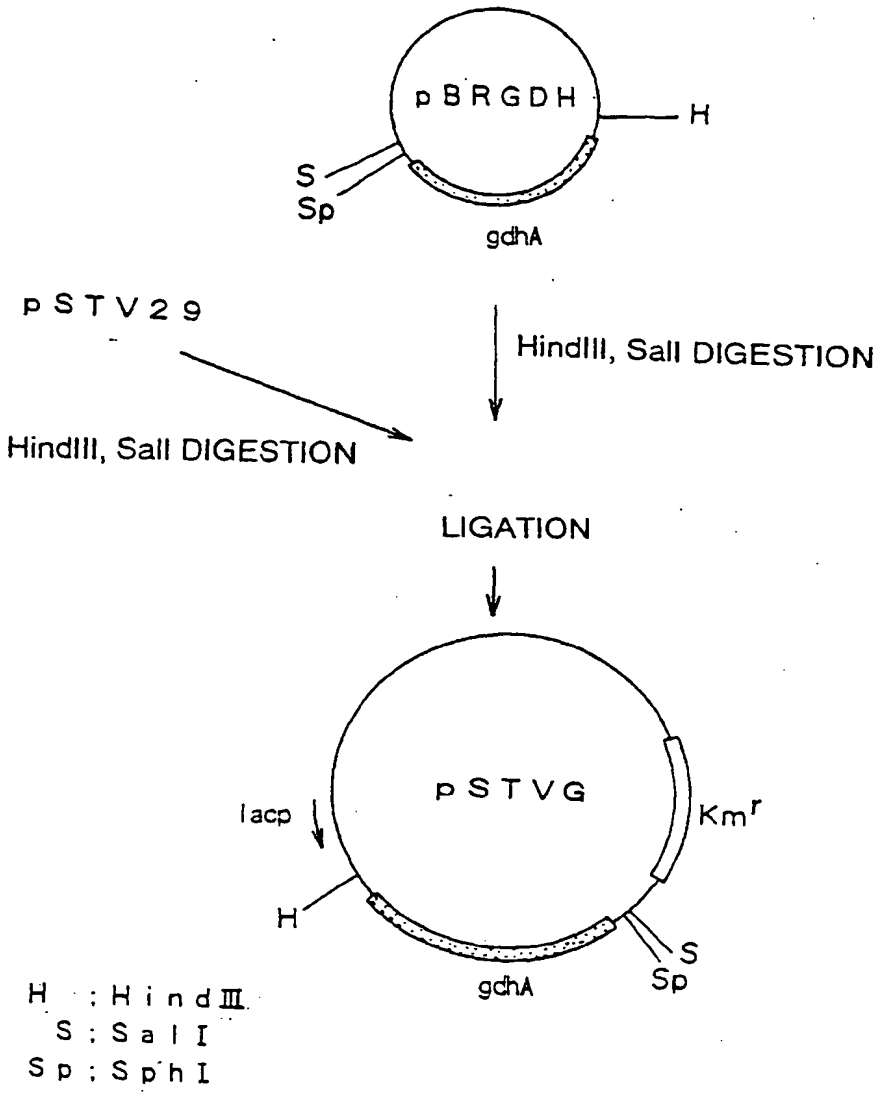
E
pMWCP
gltA
ppc
X
S
XbaI
DIGESTION
T4 DNA POLYMERASE
pBRGDH
H
Sp
gdhA
HindIII,SphI
DIGESTION
T4 DNA POLYMERASE
COLLECTION
LIGATION
E
S
gltA
pMWCPG
ppc
gdhA
S
S;SalI
E;EcoRI
Sp;SphI
X;XbaI
H;HindIII

FIG. 1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H
S
Sp
gdhA
pSTV29
HindIII, SalI DIGESTION
HindIII, SalI DIGESTION
LIGATION
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lacp
Km^r
H
S
Sp
gdhA
H : HindIII
S : SalI
Sp : SphI

FIG. 2

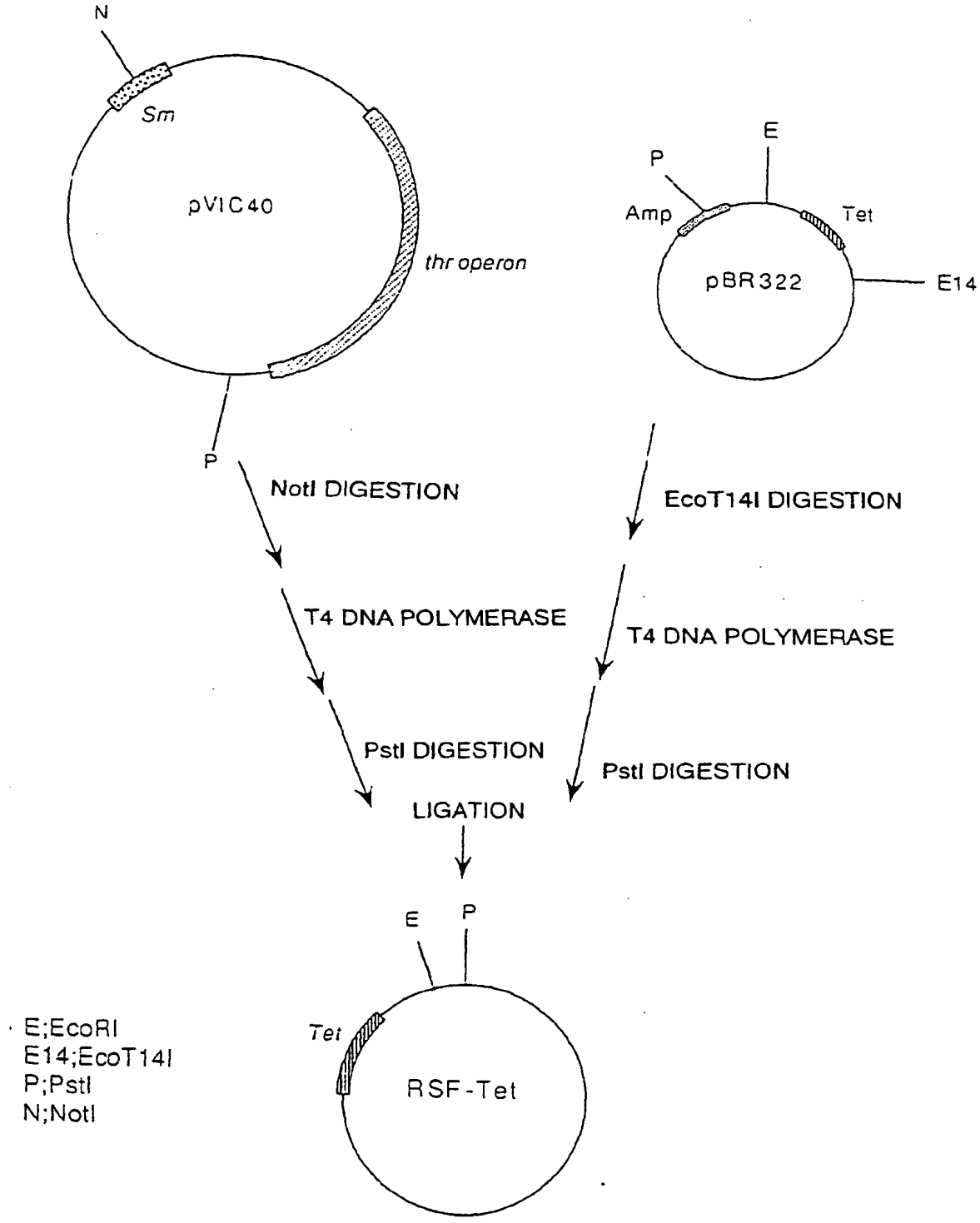
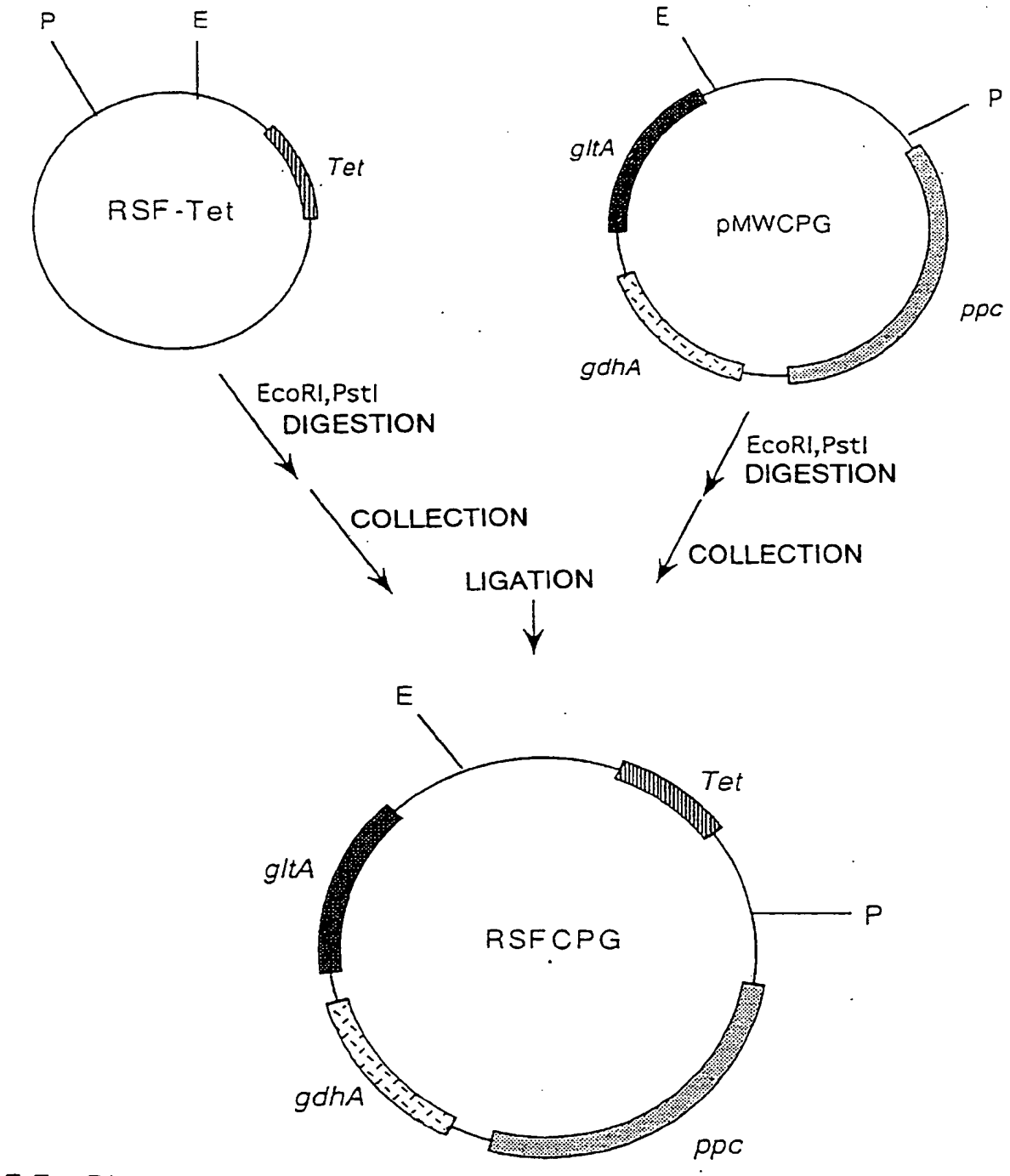

N
Sm
pVIC40
thr operon
P
NotI DIGESTION
T4 DNA POLYMERASE
PstI DIGESTION
E
P
Amp
Tet
pBR322
E14
EcoT14I DIGESTION
T4 DNA POLYMERASE
PstI DIGESTION
LIGATION
E
P
Tet
RSF-Tet
E;EcoRI
E14;EcoT14I
P;PstI
N;NotI

FIG. 3



E;EcoRI
P;PstI

FIG. 4

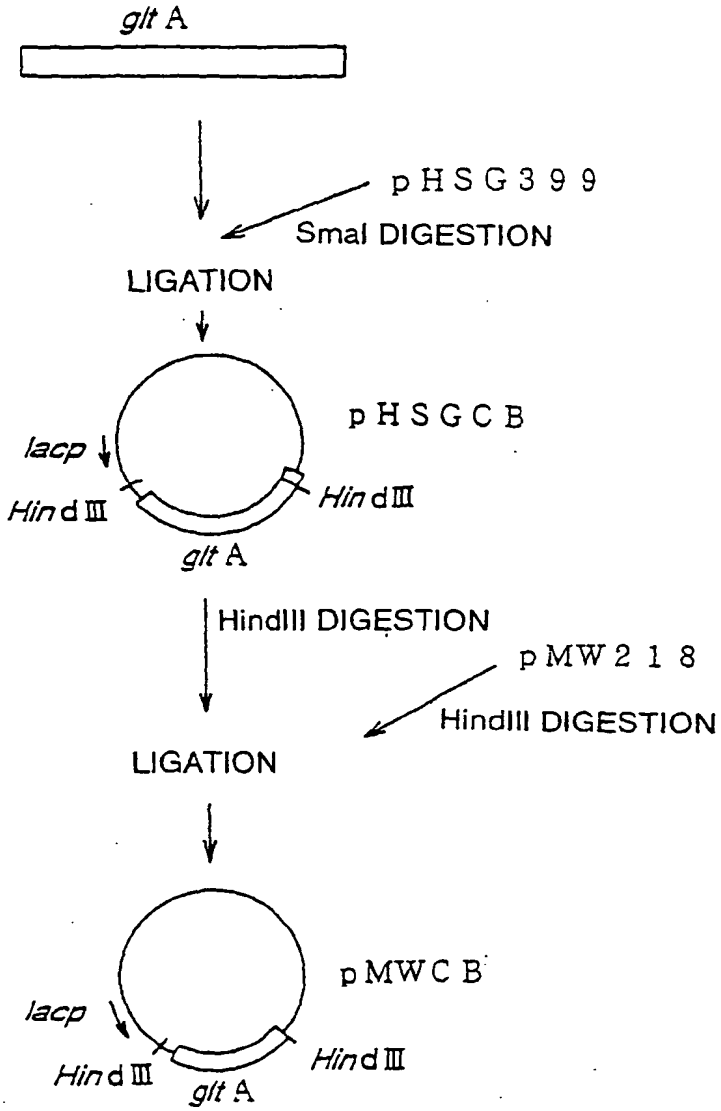
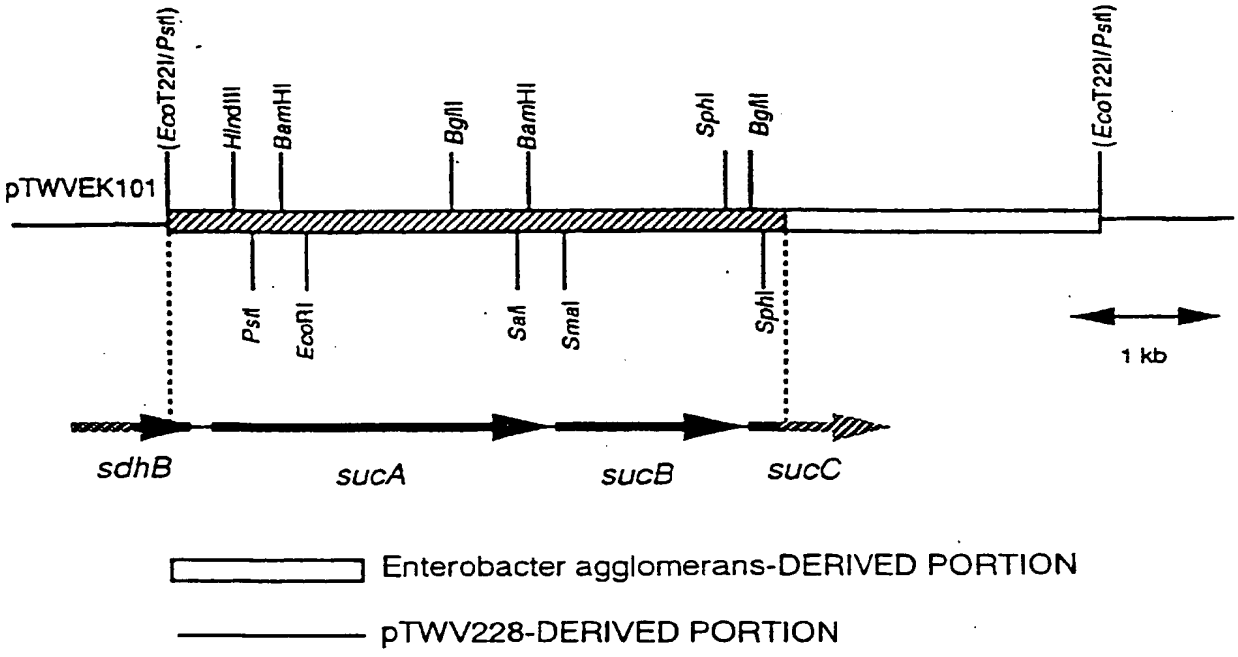

glt A
pHSG399
SmaI DIGESTION
LIGATION
pHSGCB
lacp
Hind III
Hind III
glt A
HindIII DIGESTION
pMW218
HindIII DIGESTION
LIGATION
pMWCB
lacp
Hind III
Hind III
glt A

FIG. 5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(EcoT22I/PstI)
HindIII
BamHI
BglII
BamHI
SphI
BglII
(EcoT22I/PstI)
PstI
EcoRI
SalI
SmaI
SphI
1 kb
sdhB
sucA
sucB
sucC
Enterobacter agglomerans-DERIVED PORTION
pTWV228-DERIVED PORTION

FIG. 6

[88.0% / 935 aa]

```
   1' MQNSAMKPWLDSSWLAGANQSYIEQLYEDFLTDPDSVDAVWRSMFQQLPGTGVKPEQFHS
   1" MQNSALKAWLDSSYLSGANQSWIEQLYEDFLTDPDSVDANWRSTFQQLPGTGVKPDQFHS

  61' ATREYFRRLAKDASRYTSSVTDPATNSKQVKVLQLINAFRFRGHQEANLDPLGLWKQDRV
  61" QTREYFRRLAKDASRYSSTISDPDTNVKQVKVLQLINAYRFRGHQHANLDPLGLWQQDKV

 121' ADLDPAFHDLTDADFQESFNVGSFAIGKETMKLADLFDALKQTYCGSIGAEYMHINNTEE
 121" ADLDPSFHDLTEADFQETFNVGSFASGKETMKLGELLEALKQTYCGPIGAEYMHITSTEE

 181' KRWIQQRIESGASQTSFSGEEKKGFLKELTAAEGLEKYLGAKFPGAKRFSLEGGDALVPM
 181" KRWIQQRIESG--RATFNSEEKKRFLSELTAAEGLERYLGAKFPGAKRFSLEGGDALIPM

 241' LREMIRHAGKSGTREVVLGMAHRGRLNVLINVLGKKPQDLFDEFSGKHKEHLGTGDVKYH
 239" LKEMIRHAGNSGTREVVLGMAHRGRLNVLVNVLGKKPQDLFDEFAGKHKEHLGTGDVKYH

 301' MGFSSDIETEGGLVHLALAFNPSHLEIVSPVVMGSVRARLDRLAEPVSNKVLPITIHGDA
 299" MGFSSDFQTDGGLVHLALAFNPSHLEIVSPVVIGSVRARLDRLDEPSSNKVLPITIHGDA

 361' AVIGQGVVQETLNMSQARGYEVGGTVRIVINNQVGFTTSNPKDARSTPYCTDIGKMVLAP
 359" AVTGQGVVQETLNMSKARGYEVGGTVRIVINNQVGFTTSNPLDARSTPYCTDIGKMVQAP

 421' IFHVNADDPEAVAFVTRLALDYRNTFKRDVFIDLVCYRRHGHNEADEPSATQPLMYQKIK
 419" IFHVNADDPEAVAFVTRLALDFRNTFKRDVFIDLVSYRRHGHNEADEPSATQPLMYQKIK

 481' KHPTPRKIYADRLEGEGVASQEDATEMVNLYRDALDAGECVVPEWRPMSLHSFTWSPYLN
 479" KHPTPRKIYADKLEQEKVATLEDATEMVNLYRDALDAGDCVVAEWRPMNMHSFTWSPYLN

 541' HEWDEPYPAQVDMKRLKELALRISQVPEQIEVQSRVAKIYNDRKLMAEGEKAFDWGGAEN
 539" HEWDEEYPNKVEMKRLQELAKRISTVPEAVEMQSRVAKIYGDRQAMAAGEKLFDWGGAEN

 601' LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVVHNQANGSTYTPLHHIHNSQGEFKVWDSV
 599" LAYATLVDEGIPVRLSGEDSGRGTFFHRHAVIHNQSNGSTYTPLQHIHNGQGAFRVWDSV

 661' LSEEAVLAFEYGYATAEPRVLTIWEAQFGDFANGAQVVIDQFISSGEQKWGRMCGLVMLL
 659" LSEEAVLAFEYGYATAEPRTLTIWEAQFGDFANGAQVVIDQFISSGEQKWGRMCGLVMLL

 721' PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRPLVVMSP
 719" PHGYEGQGPEHSSARLERYLQLCAEQNMQVCVPSTPAQVYHMLRRQALRGMRRPLVVMSP

 781' KSLLRHPLAISSLDELANGSFQPAIGEIDDLDPQGVKRVVLCSGKVYYDLLEQRRKDEKT
 779" KSLLRHPLAVSSLEELANGTFLPAIGEIDELDPKGVKRVVMCSGKVYYDLLEQRRKNNQH

 841' DVAIVRIEQLYPFPHQAVQEALKAYSHVQDFVWCQEEPLNQGAWYCSQHHFRDVVPFGAT
 839" DVAIVRIEQLYPFPHKAMQEVLQQFAHVKDFVWCQEEPLNQGAWYCSQHHFREVIPFGAS

 901' LRYAGRPASASPAVGYMSVHQQQQQDLVNDALNVN
 899" LRYAGRPASASPAVGYMSVHQKQQQDLVNDALNVE
```

FIG. 7

[88.2% / 407 aa]

```
    1' MSSVDILVPDLPESVADATVATWHKKPGDAVSRDEVIVEIETDKVVLEVPASADGVLEAV
    1" MSSVDILVPDLPESVADATVATWHKKPGDAVVRDEVLVEIETDKVVLEVPASADGILDAV

   61' LEDEGATVTSRQILGRLKEGNSAGKESSAKAESNDTTPAQRQTASLEEEESSDALSPAIRR
   61" LEDEGTTVTSRQILGRLREGNSAGKETSAKSEEKASTPAQRQQASLEEQNNDALSPAIRR

  121' LIAEHNLDAAQIKGTGVGGRLTREDVEKHLANKPQAEKAAAPAAGAATAQQPVANRSEKR
  121" LLAEHNLDASAIKGTGVGGRLTREDVEKHLAKAPAKE--SAPAAAAPAAQPALAARSEKR

  181' VPMTRLRKRVAERLLEAKNSTAMLTTFNEINMKPIMDLRKQYGDAFEKRHGVRLGFMSFY
  179" VPMTRLRKRVAERLLEAKNSTAMLTTFNEVNMKPIMDLRKQYGEAFEKRHGIRLGFMSFY

  241' IKAVVEALKRYPEVNASIDGEDVVYHNYFDVSIAVSTPRGLVTPVLRDVDALSMADIEKK
  239" VKAVVEALKRYPEVNASIDGDDVVYHNYFDVSMAVSTPRGLVTPVLRDVDTLGMADIEKK

  301' IKELAVKGRDGKLTVDDLTGGNFTITNGGVFGSLMSTPIINPPQSAILGMHAIKDRPMAV
  299" IKELAVKGRDGKLTVEDLTGGNFTITNGGVFGSLMSTPIINPPQSAILGMHAIKDRPMAV

  361' NGQVVILPMMYLALSYDHRLIDGRESVGYLVAVKEMLEDPARLLLDV
  359" NGQVEILPMMYLALSYDHRLIDGRESVGFLVTIKELLEDPTRLLLDV
```

FIG. 8

[95.1% / 41 aa]

```
    1' MNLHEYQAKQLFARYGMPAPTGYACTTPREAEEAASKIGAG
    1" MNLHEYQAKQLFARYGLPAPVGYACTTPREAEEAASKIGAGPWVVKCQVHAGGRGKAGGV
```

FIG. 9

[97.4% / 39 aa]

```
    1'                          AFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMLLQRSA
  181" FLIDSRDTETDSRLDGLSDAFSVFRCHSIMNCVSVCPKGLNPTRAIGHIKSMLLQRNA
```

FIG. 10

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(71) Applicant: **Ajinomoto Co., Inc.**
Tokyo (JP)

(72) Inventors:
- **Izui, Hiroshi c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Ono, Eiji c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Matsui, Kazuhiko c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Moriya, Mika c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Ito, Hisao c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)
- **Hara, Yoshihiko c/o Ajinomoto Co.,Inc.**
Kawasaki-ku, Kawasaki-shi, Kanagawa-ken (JP)

(74) Representative: **Strehl Schübel-Hopf & Partner**
Maximilianstrasse 54
80538 München (DE)

(54) L-glutamic acid-producing bacterium and method for producing l-glutamic acid

(57) L-Glutamic acid is produced by culturing in a liquid culture medium a microorganism belonging to the genus *Enterobacter* or *Serratia* and having an ability to produce L-glutamic acid, which increases in an activity of enzyme catalyzing a reaction for L-glutamic acid biosynthesis, or which decreases in or is deficient in an activity of an enzyme catalyzing a reaction branching from a pathway for L-glutamic acid biosynthesis and producing a compound other than L-glutamic acid, and collecting produced L-glutamic acid from the culture medium.

EP 0 952 221 A3

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EUROPEAN SEARCH REPORT

Application Number
EP 99 10 5508

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.6)
X,D	EP 0 670 370 A (AJINOMOTO KK) 6 September 1995 (1995-09-06) * the whole document *	1,2,4,6	C12N15/53 C12N15/60 C12N1/21 C12N1/20
X	WO 97 08294 A (AJINOMOTO KK ;MATSUI KAZUHIKO (JP); HUKASE KUMIKO (JP); TSUJIMOTO) 6 March 1997 (1997-03-06) * the whole document *	1,2,4,6	C12P13/14 //(C12N1/20, C12R1:01, 1:425)
E	EP 1 078 989 A (AJINOMOTO KK) 28 February 2001 (2001-02-28) * the whole document *	1-6	

TECHNICAL FIELDS SEARCHED (Int.Cl.6)

C12N
C12P

The present search report has been drawn up for all claims

Place of search	Date of completion of the search	Examiner
THE HAGUE	20 July 2001	Oderwald, H

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This annex lists the patent family membersrelating to the patent documents cited in the above-mentioned European search report. The members are as contained in the European Patent Office EDP file on
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20-07-2001

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 0670370 A	06-09-1995	JP 7203980 A	08-08-1995
		BR 9500052 A	03-10-1995
		CN 1128295 A	07-08-1996
		US 5573945 A	12-11-1996
WO 9708294 A	06-03-1997	BR 9610016 A	06-07-1999
		CN 1193343 A	16-09-1998
		DE 19681532 T	03-12-1998
		FR 2738014 A	28-02-1997
		US 6110714 A	29-08-2000
EP 1078989 A	28-02-2001	AU 5345200 A	22-02-2001
		BR 0003695 A	05-06-2001
		CN 1292421 A	25-04-2001
		PL 341974 A	26-02-2001